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Fish Collagen – Molecular Structure After Thermal Treatment

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Abstract
Modern medicine widely uses exogenous collagen as a good material for tissue regeneration, also as a natural substrate for cell attachment and proliferation, used to create dressings and to support the treatment of burn and diabetic wounds, or finally as a source of amino acids in the form of a dietary supplement. Collagen is a safe material that has high biocompatibility and biodegradability as well as good cell adhesion. Due to the possibility of transferring Creutzfeld-Jacob's disease (Bovine Spongiform Encephalopathy) from animals to the human body, interest in collagen from fish is currently increasing. The collagen we examined was derived from the skin of the silver carp fish (Hypophtalmichthys molitrix) and was obtained by the method of hydration in an aqueous lactic acid solution. The topography of the test sample was performed with the AFM method, showing its fibrillar structure with dimensions equivalent to those given in iterature. Raman spectroscopy was used to study fish collagen using a Renishaw Ramanscope with a helium-neon laser at a wavelength of 633 nm. Analysis of Raman spectra allowed to determine the content of amino acids in collagen, as well as glycine, proline and hydroxyproline. It also showed the native nature of the material at 20 °C. The partial renaturation of the secondary structure of this material heated to about 85 °C and cooled was also proved. Raman spectroscopy has been presented as an effective method for testing biopolymers.

Key words: *fish collagen gel, biopolymers, Raman spectroscopy*

Introduction

Collagen is the most abundant protein in living organisms, providing the principle structure and mechanical support for several tissues. In the human body it accounts for about 20% of all proteins, being the main component of connective tissue, skin, tendons and bone [1]. Collagens are a group of specific proteins, being the main component of the extracellular matrix of connective tissues. Over twenty types of collagens have been identified [2]. The basic collagen molecule consists of three left-handed polypeptide chains called alpha-chains (α-helix). Interchain hydrogen bonds make those three levorotatory helixes coaxe together around a common axis, forming a dextrorotatory helix (triple-helix or major helix). Although the composition and sequence of amino acids vary depending on the type of collagen, they always contain large amounts of glycine and amino residues (imino acid) of proline and hydroxyproline. A typical amino acid sequence in collagen is (Gly – X – Y), where X is most often proline and Y – hydroxyproline. Glycine always occupies the third position in the polypeptide chain.

Collagen molecules are widely used in the field of tissue engineering, especially as cell culture hydrogels [3] and as 3D scaffolds for the analogues of biological systems [4, 5], or in the production of new generation dressings [6] in implantology [7], and even in the textile industry [8, 9].

In this work we performed an investigation on the fish collagen gel FCG. The increasing interest in collagen derived from fish skin is associated with mastering the technique of obtaining native collagen by acid hydration [10] and the threat resulting from exposure to the Creutfeld-Jacob or BSE diseases, related to the possibility of transferring prions through bovine collagen. This method allows to obtain the native form of collagen in the final product. Collagen obtained in this way can be used not only in the form of a gel or solution, but also in a solid form, replacing the bovine collagen used until now.

Experimental approaches

Materials

Collagen was derived from the skin of a silver carp fish (*Hypophtalmichthys molitrix*), and was obtained by the method of hydration in an aqueous lactic acid solution [11]. The skins were firstly cleaned and then placed in a solution of lactic acid (1.5% V/V concentration). The hydration process was carried out in glass containers in a temperature range from 15 to 20 °C for 24 to 48 hours, accompanied by running visual control of the process. Next the collagen was purified from cell debris, pigments and remains of the acid solution via the filtration process using silk filters of increasing density. Natural silk filters have a structure similar to that of collagen, which prevents damage to the collagen structure. As obtained, the collagen gel (FCG) was deposited on plates of CaF_2 for examination by Raman spectroscopy. For the AFM method, the collagen gel was diluted with distilled water (1:100 w/w) and deposited on a silicon wafer. Collagen remaining in an aqueous solution can undergo denaturation at 41 °C, while collagen fibers, when in alignment, exhibit a rather broad phase transition, characterised by shrinkage at elevated temperatures. The center of this transition is at approximately 55 °C [12]. For examination of the denatured state of collagen, labelled FCG2, a temperature of 85 °C was selected because denaturation takes place below this. Safandowska [13] in DSC measurements for the same material indicated that the denaturation temperature is about 80 °C. Collagen FCG2 was quickly heated to a temperature of 85 °C and then cooled down. All the Raman spectra were recorded after 24 h at 20 °C.

Methods

AFM

Atomic force microscopy (AFM) measurements were performed using an Icon Scanning Probe Microscope (Bruker, USA) in the contact mode. Each topography image was obtained at 0.2 Hz and lines at 512. All AFM data analysis was performed by Gwyddion software. AFM can be used to obtain topology images and other qualitative and quantitative information, such as mechanical properties, in a non-destructive way, from a wide range of samples, including biological ones. AFM operates by measuring forces

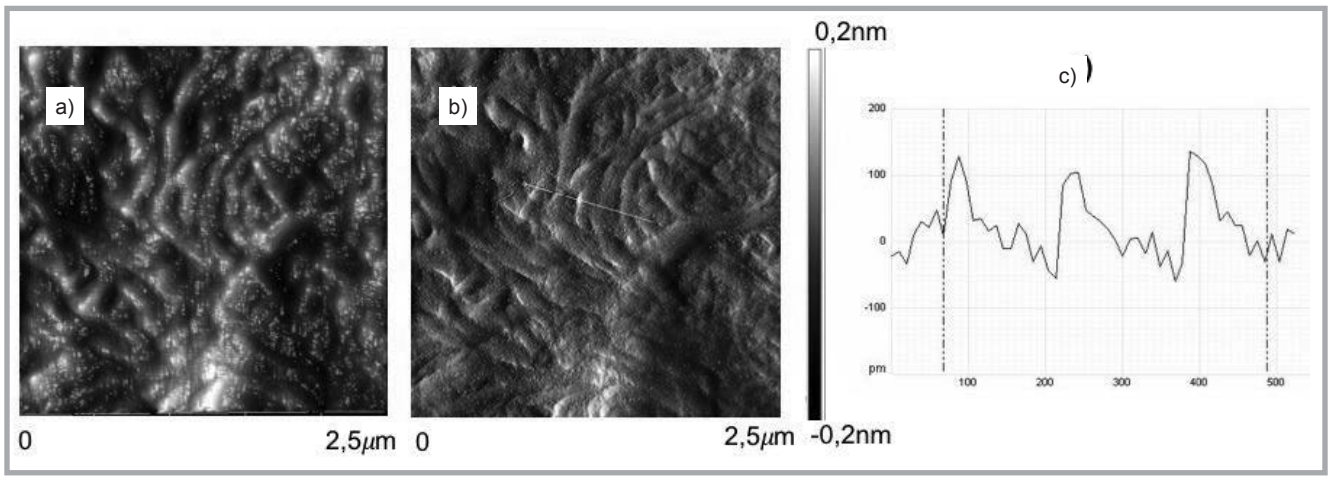

Figure 1. *AFM topography images of FCG: a) 3-D image, b) AFM-Amplitude topography. A white line cuts three adjacent fibrils; c) section analysis along the white line from part b).*

between a probe and the sample and offers extremely high resolution at the nanometer level, without special treatment, such as dehydration, labeling or coating, or vacuum conditions of the specimen. Moreover it operates efficiently in samples embedded in water or buffers as well as on live cells;, and even detects molecules at the single-molecule level.

Raman spectroscopy

Raman spectra of the fish collagen were recorded in air at a temperature 20 °C using a Confocal Raman Microspectrometer (Renishaw Ramanscope 1000 spectrometer, UK) with a 50x/0.75 microscope objective (LEICA) equipped with an He-Ne laser delivering 5 mW of power at $\lambda = 633$ nm. Raman spectra were collected by WiRE™3.3 software attached to the instrument. For molecular vibration to generate a Raman band, there must be a related polarisation change in the chemical bond, so that there is a corresponding distortion of electron density near the vibrating nuclei. Raman spectroscopy is now a versatile tool in protein science and biotechnology that gives structural information about complex solid systems. The most useful Raman bands in determining the secondary structure of protein are amide I, amide II, and amide III bands. As proteins are large polypeptides, their vibrational spectra consist of a complex set of overlapping bands.

Results and discussion

AFM imaging

Analysis of the structure and size of the FCG material examined confirmed the presence of collagen fibrils in the test sample. We observed collagen fibers with an average cross-sectional diameter of 156±31 nm (***Figure 1***), which are likely to be an intermediate form in the creation of larger collagen fibers from tropocollagen, seen in natural tissue [14]. The fibrils are oriented in different directions, which could be related to the preparation via filtering with silk filters, and could be a helpful feature of using collagen as a natural substrate for cell attachment and proliferation in wound healing. We can state that the banding pattern structure of collagen is not visible due to the strong hydration of collagen fibers [15]. The image is similar to that obtained from an electron microscope for fish swim bladder collagen, fibers of which strongly swelled under the action of acetic acid [16].

Raman spectra

Because proteins are huge polypeptides consisting of hundreds of amino acids, their Raman spectra are composed of many overlapping bands. In addition, there are only a few proteins with a clearly defined structure, for example only α-helix and β-sheet are without any other structure involvement. The primary structure of proteins is a sequence of repeating amino acids. The CO-NH peptide bonds between individual proteins give their contribution to the Raman spectrum in the form of amide bands from I to VII and also A & B. The relatively high intensity amide I and amide III bands carry information about the internal structure of the protein and its conformation. The largest contribution to amide I is made by the stretching vibrations of the C-O carbonyl group of peptide (80%). The rest is made up of C-N stretching vibrations and bending vibrations of N-H groups [17]. Amide III is mainly composed of C-N group stretching vibrations (40%), N-H group bending vibrations (30%) and CH_3-C group stretching vibrations (20%) [17, 18]. Protein conformation is also determined by the interaction of the backbone with the protein's side chain. Such interactions include intramolecular hydrogen bonds between the oxygen atom of the carbonyl group of each peptide bond and the hydrogen atom of the amino group of the fourth consecutive amino acid in the backbone sequence. This creates a hydrogen bond almost parallel to the main axis of the helix [19].

Collagen FCG

Fingerprint region

A Raman spectrum provides a "fingerprint" that is unique to the material. The fingerprint region for Raman spectroscopy is in range of 400-1800 cm^{-1}. A representative Raman spectrum of FCG is shown in ***Figures 2.a*** and ***2.b***. The range 920-937 cm^{-1} is attributed to stretching vibrations of C-N groups in the proline. The range 855-887 cm^{-1} is derived from proline and hydroxyproline or the tyrosine side chain. Proline and hydroxyproline represent around 20% of the total number of amino acids in the collagen molecule. The presence of hydroxyproline is characteristic only for collagen – it is not found in other proteins. Raman markers of glycine are very weak and are hidden in the total spectrum (~ 1345 cm^{-1}, CH_2 twist or rock vibration [20]. Leucine and lysine together constitute about 5% of the amino acids contained in fish collagen [22]. Overman and Thomas [23] showed that the interval 730-745 cm^{-1} may be a α-helix marker, tentatively

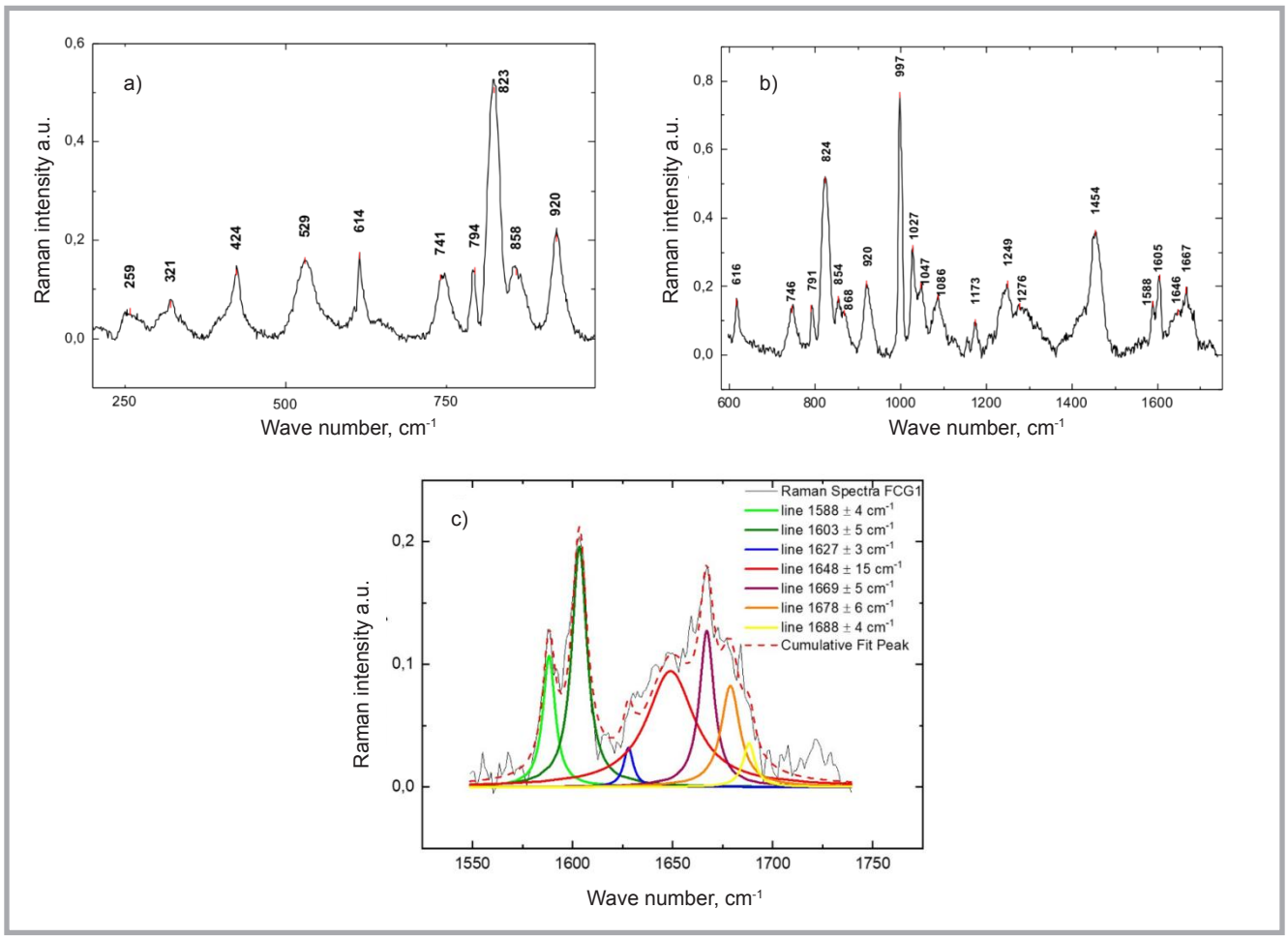

Figure 2. *Raman spectra for collagen FCG in the domain 250-1800 cm^{-1}, divided into two overlapping parts: a) 250-950 cm^{-1} and b) 600-1800 cm^{-1}, including the "fingerprint region"; c) fitting process of the amide I band.*

assigned to the carbonyl in-plane bending mode. Alanine is represented in the Raman spectrum by the strong line at 1455 cm^{-1} of methyl group CH_3 bending vibrations. The remaining amino acids in collagen occur in trace quantities. Tryptophan and cysteine do not exist in the collagen molecule. Our spectra show this with the lack of lines in areas 1550-1560 cm^{-1} and 2526-2560 cm^{-1}, respectively. ***Table 1*** presents wavenumbers of the bands observed and their assignment. It can be noticed that the composition of collagen FCG revealed by means of Raman spectra agrees with the amino acid analysis performed by Gauza [24] and Safandowska [13] for the same material, in which the presence of hydroxyproline was tested separately, due to its large influence on the stabilisation of collagen fibrils [25] in the position in which Hyp occurs naturally.

Region 2600-3200 cm^{-1}

The band assigned to CH_2 and CH_3 stretching vibrations is observed near 2940 cm^{-1} and consists of quartet lines: mainly 2940 cm^{-1} ($\nu_{as}CH_2$, ν_sCH_3) and three shoulders – 2830 cm^{-1} (ν_sCH_2), 2884 cm^{-1} (ν_sCH_2) and 2988 cm^{-1} ($\nu_{as}CH_3$). This quartet was predicted in collagen by Ramachandran [26] and is observed in tripeptide chains like polyproline II [27]. ***Figure 3*** presents these spectra for FCG (black line) and FCG2 (red line), for comparison. The spectra did not change after heating, because they are connected with the primary structure of collagen.

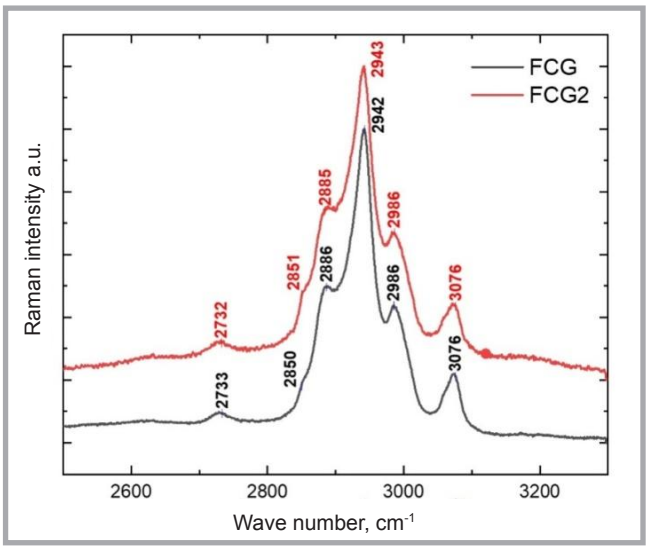

Figure 3. *Raman spectra for collagens FCG and FCG2 in the range 2600-3200 cm^{-1}.*

Amide bands

For determining the secondary structure of proteins (α-helix, β-sheet, random coil), amide I and amide III bands

Table1. *Assignment of vibration bands of Raman spectra of collagen FCG.* ***Note:*** *ν – stretching mode, δ – bending mode, sh – shoulder.*

Peak, cm^{-1}	Assignement		Refs
Primary			
529/531	S – S	TGG	[31]
616		phenylalanine	[40]
745	δ(CCO) CH_2rocks	Amide IV, carbonyl group Lysine, leucine	[23] [20]
824	ν(CC)	tyrosine	[40]
858	ν(CC), δ(CCH)	Proline, hydroxyproline, tyrosine	[41], [40]
875sh	ν(CC), δ(CCH)	hydroxyproline	[41]
920	ν(CC)	proline	[42]
997,1027	ν(CC)	phenylalanine	[40]
1087	ν(CH_3)	alanine	[20]
1455	ν(CH_2, CH_3)	Amino acid side chain, alanine	[20]
1586, 1606		phenylalanine	[40]
Quartet (2940,1840sh, 2884sh, 2988sh)	ν(CH_2, CH_3)	Amino acid aliphatic side chain	[12], [31], [27]
3080	ν(C=CH)	Amide B	[27]
Secondary			
1247	ν(CN), δ(NH)	Amide III, random coil	[30]
1282	ν(CN), δ(NH)	Amide III, α-helix	
1665	ν(C=O)	Amide I	

are used. Amide I has its center around 1650-1680 cm^{-1}, and involves mainly C=O stretching vibrations and partly the C–N stretching, Cα-C-N bending and N-H in-plane bending of peptide groups. Generally α-helical proteins show an amide I center around 1650-1658 cm^{-1}, that the random-coil structure of proteins is related to the spectral range 1660-1665 cm^{-1}, and that β-sheet type proteins have a center around 1665-1680 cm^{-1} [28]. The amide III mode comprises of C-N stretching and N-H in-plane bending vibrations of the peptide bond, as well as Cα-C stretching and C=O in-plane bending. The Raman band of amide III for proteins is between 1200-1300 cm^{-1}; the β-sheet is around 1220-1240 cm^{-1}; the random coil is located near 1240-1245 cm^{-1}, and the α-helix covers the range of 1260-1290 cm^{-1} [21]. However, it is difficult to distinguish the disordered form from the β structure because the amide I band of β-turns overlaps those of α-helices and β-sheets, making assignment of this component solely from amide I difficult. When used in combination with the amide III band, their assignment may permit a distinction between β and the disordered structure [29]. ***Figure 2.c*** presents the amide I band for FCG. Fitting of the Raman spectra was performed using the OriginLab program with the Lorenz function, where a half of the FWHM is a measure of the resolution for spectral lines. The secondary structure of FCG is mixed and composed in comparable proportions of the random coil 1669 cm^{-1} band, α-helix 1648 cm^{-1} band, and β-sheet 1678 cm^{-1} band [30]. The weak line at 1688 cm^{-1} can be assigned to β-turn or the unordered structure [31]. The amide I spectrum is completed by the line at 1627 cm^{-1}, which, according to the latest reports [32], can be assigned to the triple helix in the collagen molecule. In this way we prove the native nature of the material being examined. Large parts of the random coil structure can be caused by the collagen production method, where the material is repeatedly filtered through micron filter silk. As a result, long chains of collagen were divided into smaller fragments with unfolded ends, which resulted in an increase in the signal in the band of the random coil. At the edge of the spectrum we have two lines responsible for skeletal vibrations of collagen, described in ***Table 1***. Amide III for FCG is built by the strong line at 1249 cm^{-1} , corresponding to the random coil, with the left side band at about 1220 cm^{-1} related to the β-sheet and the band at 1276 cm^{-1} to α-helix conformation.

Triple helix

The most important to prove the presence of a triple helix in collagen is an analysis of the amide I band. There are some other markers important to confirm the triple helix structure. One of the most helpful side-chain markers is the tyrosine Fermi doublet connected with lines at ~855 cm^{-1} and ~825 cm^{-1}, arising from an interaction between the ring breathing fundamental and the overtone of C–C–O deformation in the *para*–substituted aromatic ring of tyrosine. In the native protein, tyrosine is buried inside the protein molecule, while for unfolded protein tyrosine residues are exposed on the molecular surface and interact with the hydrophilic medium [33]. The Raman intensity ratio I_{850}/I_{825} indicates how strong the hydrogen bond of the tyrosine phenoxyl group is [34]. When the phenoxyl proton is the donour of a strong hydrogen bond, I_{850}/I_{825} = 0.30, when phenoxyl oxygen is the acceptor of a strong hydrogen bond, I_{850}/I_{825} = 2.5, and when the phenoxyl group is both a donor and acceptor, as for a solvent-exposed tyrosine, I_{850}/I_{825} is between (0.9-1.45) [28]. The intensity ratio I_{850}/I_{825} calculated from the Raman spectrum for the FCG presented in ***Figure 2.a***, possesses a value of I_{850}/I_{825}=0.31, which is the minimum value for this coefficient, where tyrosine is the most buried in its structure. The next marker is connected with the band at 531 cm^{-1}, assigned to disulfide bridges S-S, which play an important role in maintaining the tertiary structure of proteins [30]. The S-S stretching band is sensitive to the conformation of the disulfide bridge, where the ν_{SS} band appears at around 523-528 cm^{-1} the conformation is *trans-gauche-gauche* (TGG) [35]. For FCG we observe this band at around 529 cm^{-1}.

Denaturation and renaturation of the collagen FCG2

Denaturation of the protein molecules means the destruction of its tertiary structure and the loss of its biological character and activity. Here we investigated thermal denaturation. The thermal stability of fish collagen depends on several factors like the physiological temperature of the fish (fish living in warmer waters have a higher denaturation temperature) and high content of hydroxyproline, which stabilises the triple helix. For collagen films there are some other parameters such as the solvent, ionic strength, pH and humidity, and hence the analysis become more complex [36].

In protein like collagen, the destruction of the tertiary structure means the breaking of intra- and intermolecular cross-links. Kühn and his group investigated acid-soluble collagen (pH 3.7) after complete denaturation and proved its renaturation after cooling by 50% [37]. They showed the possibility of regeneration of real native molecules from fully denatured tropocollagen, i.e. completely separated into subunits. In our experiment, af-

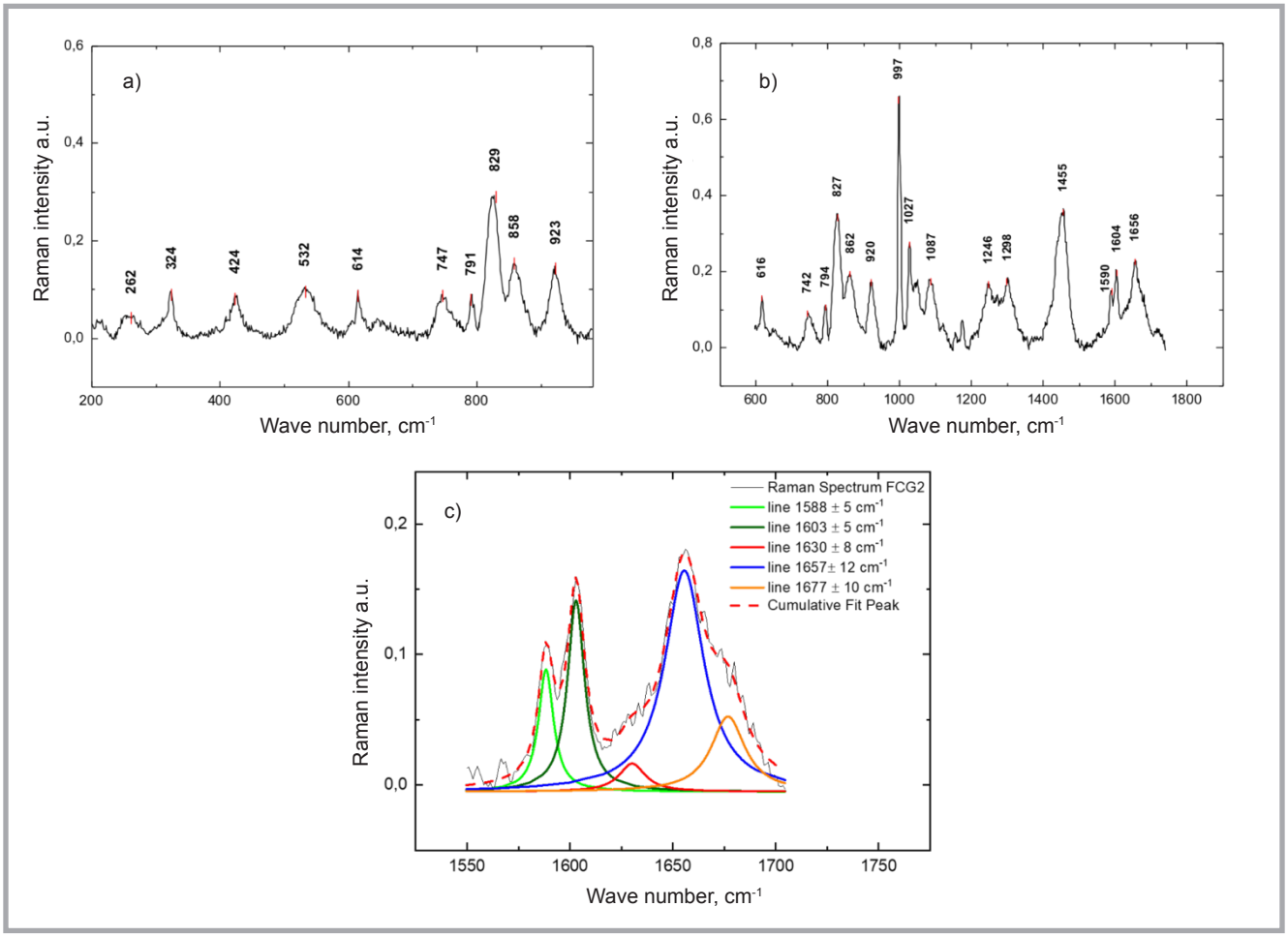

Figure 4. *Raman spectra for collagen FCG2 in the domain 250-1800 cm^{-1}, divided into two overlapping parts: a) 250-950 cm^{-1} and b) 600-1800 cm^{-1}, including the "fingerprint region"; c) fitting process of the amide I band.*

ter heating the FCG sample to a temperature of 85 °C , the collagen was cooled, and Raman spectra were taken after 24 h at a temperature of 20 °C. The centers and intensity of the line connected with backbone vibrations do not change within the measuring accuracy, which can be observed in ***Figures 4.a*** and ***4.b***. It shows that the primary structure of collagen – the amino-acid sequence, was not disrupted during this heating. On the other hand, a major change in the secondary structure can be noticed. In the amide I band, the main peak has a narrow shape and its center is shifted to the area of lower frequency associated with the α-helix. After the fitting process, the amide I band is comprised of the main line at 1657 cm^{-1}, assigned to the α-helix structure, the line at 1677 cm^{-1} ,connected with the β-sheet structure, and a small peak at 1630 cm^{-1}, which was previously associated with the existence of a triple helix [32] (***Figure 4.c***). The line at 1657 cm^{-1} is characteristic of denatured collagen acid solution, while the collagen solid phase after denaturation has a maximum peak at 1672 cm^{-1} [38]. It is interesting that the lines at 1588 cm^{-1} and 1603 cm^{-1} did not change their positions or intensity for both the FCG and FCG2 samples. This shows that the primary structure of collagen was not disrupted by the heating. In the amide III band one can observe a large change associated with the appearance of a strong peak at 1298 cm^{-1} on the side of the amide III band which is responsible for α-helix. For FCG2 we calculated the ratio $I_{850}/I_{825} = 0.6$ (***Figure 4.a***). The I_{850}/I_{825} ratio increased, which indicates a weaker hydrogen bond of the tyrosine phenoxyl group [34], but still below the value determined, no matter whether the tyrosine residues in the collagen are exposed (0.9-1.45). The flattening and widening of the band at 531 cm^{-1} may indicate the weakness of the disulfide bonds.

Conclusions

In this work we investigated fish collagen obtained by acid hydration. AFM studies showed the fibrous structure of this material, with dimensions corresponding to those of collagen fibrils described in the literature. These fibrils represent a transient form between the collagen molecule and the mature form in the cell. It has been demonstrated that this form of collagen has the ability to induce the production of collagen endogenous in fibroblasts [39], which promotes wound healing. We pointed to the fact that fiber swelling may be related to the acid hydration process and the appearance of large polar regions around the collagen molecules.

Raman spectroscopy has been presented as an effective method for testing biopolymers, showing the composition of the material with strands characteristic of collagen tested, especially proline and hydroxyproline. The analysis of amides I and III made it possible to determine the secondary structure of the mixed collagen FCG (α-helix, β-sheet and random coil) and indicated its native character and biological activity. This was also confirmed by the analysis of disulfide bonds and the tyrosine doublet. Thus it can be concluded that the acid hydration method of obtaining FCG did not deprive it of its native character.

Analysis of the Raman spectra for collagen subjected to thermal denaturation and cooled down to 20 °C, in which the dominant phase of the α-helix structure and a small line at 1630 cm^{-1} appeared, indicated the possibility of collagen's partial renaturation in this process and the tendency of acid collagen to self-ordering collagen threads during cooling. The folding of collagen FCG2 was confirmed by the tyrosine Fermi doublet as well. The results support the conclusion that fish collagen gel is more thermally stable than we previously thought.

These results suggest that the fish collagen materials obtained could be used in medical and textile applications.

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